SUPPLEMENTARY APPENDIX

Increments in DNA-thioguanine level during thiopurine-enhanced maintenance therapy of acute lymphoblastic leukemia

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Appendix 1: **Increments in DNA-thioguanine level during thiopurine enhanced maintenance therapy of acute lymphoblastic leukemia**
TEAM: Thiopurine Enhanced ALL Maintenance therapy

Content:

1. **Inclusion process and inclusion sites**
   Before inclusion patients had to complete four weeks of standard Methotrexate(MTX)/6-mercaptopurine (6MP) maintenance-II. When the TEAM strategy was proven tolerable for the first 19 patients, this criterion was omitted April 2018.
   Upon TEAM inclusion, white blood cell count (WBC) had to be > 1.5 $x10^9$/L, absolute neutrophil count (ANC) > 0.5 $x10^9$/L, thrombocyte count (TBC) > 50 $x10^9$/L, bilirubin < upper normal level (UNL), and coagulation factors II-VII-X > 0.5 IU/L or International Normalized Ratio (INR) < 1.5.
   In case of myelotoxicity or hepatoxicity upon screening, inclusion was postponed until the parameters had improved accordingly. In September 2017 the inclusion criteria of DNA-TG below 500 fmol/µg DNA was removed. Data from the ALL2008 MT sub-study demonstrated that the relapse hazard rate was reduced by 28% for each 100 fmol/µg DNA increment in DNA-TG (adjusted for sex, age and WBC at diagnosis) without indication of plateauing of the effect.(1)
   Patients with DNA-TG > 500 fmol/µg DNA at inclusion could therefore still benefit from participation in TEAM by further increments in DNA-TG. Further, patients with DNA-TG > 1500 fmol/µg DNA were excluded, due to a hypothetical, although not shown, association with toxicity.

   Diagnosis of sinusoidal obstruction syndrome (SOS): At least three out of five criteria needed to be fulfilled to establish diagnosis of previous SOS; (i) hepatomegaly, (ii) hyperbilirubinemia above UNL, (iii) ascites, (iv) weight gain ≥5%, and (v) thrombocytopenia (transfusion-resistant and/or otherwise unexplained by treatment).(2)

   Childhood patients were included from three pediatric oncology departments in Denmark, starting in August 2016: Rigshospitalet, University of Copenhagen, Odense University Hospital and Aarhus University Hospital. Further, one child was included from Division of Pediatric Hematology and Oncology, HUS Helsinki University Hospital, New Children's Hospital, Finland. Adult patients were all included from Department of Hematology, Rigshospitalet, University of Copenhagen, Denmark.

2. **Data Safety Monitoring**
   The TEAM study was conducted according to the general rules of the Declaration of Helsinki II and according to guidelines for Good Clinical Practice (GCP). The TEAM study was regularly monitored by the GCP Unit of the Capital Region of Denmark. Written informed consent was obtained from all participants or legal guardians. The TEAM study was approved by the Data Protection Agency, the Ethical Committee of the Capital Region of Denmark (H-3-2014-098), and the Danish Medicines Agency (2014-002248-42). Occurrence of Serious Adverse Events (SAEs), sinusoidal obstruction syndrome (SOS), and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) had to be reported within 24 hours to the Sponsor.
   The TEAM study was monitored by a Data Safety Monitoring Committee consisting of Bruce Bostrom, MD (Children's Hospitals and Clinics of Minnesota, Minneapolis, United States of America) and Martin

Stanulla, MD (Klinik für Allgemeine Pädiatrie, Universitätsklinikum Schleswig-Holstein, Kiel, Germany). The Data Safety Monitoring Committee received a safety report every sixth months with information about progression and status of the TEAM study and occurrence of SAEs. The Data Safety Monitoring Committee consistently approved the continuation of the TEAM study. An annual safety report was furthermore sent to the Danish Medicine Agency and the Ethical Committee of the Capital Region of Denmark that consistently approved the continuation of the TEAM study.

3. Maintenance therapy in the NOPHO ALL2008 protocol

Maintenance therapy was divided into two phases, maintenance-I (standard risk: weeks 20–57 following diagnosis; intermediate risk: weeks 22–59) and maintenance-II (standard risk: weeks 58–130; intermediate risk: weeks 66–130). During maintenance-I patients receive alternating therapy every fourth week with either high-dose intravenous MTX administrations or vincristine with peroral dexamethasone. High-dose intravenous MTX administrations were accompanied by intrathecal MTX, and for patients with intermediate risk ALL and central nervous system leukemia at diagnosis prednisolone and cytarabine were added to intrathecal MTX as triple intrathecal therapy (TIT).(3)
Patients were randomized to receive intramuscular pegylated-asparaginase administrations either every second (ten administrations in total) or sixth week (three administrations in total) until week 33, depending on randomization allocation as part of a NOPHO ALL2008 phase 3 trial; results hereof have been published elsewhere.(4) During maintenance-II, patients with intermediate risk ALL received intrathecal MTX at 8-week intervals, and TIT if the patient had central nervous system leukemia at diagnosis.

4. Trial outline and dose-limiting toxicities

Upon first visit, the patients' 6MP doses were reduced to 2/3rd of the 6MP dose that each patient on average had tolerated prior to inclusion. MTX dosage was unaltered. Two weeks after reduction of 6MP dose, 6-thioguanine (6TG) therapy was initiated. In April 2018, the TEAM protocol was amended to prepare for initiation of the ALLTogether protocol (EudraCT number: 2018-001795-38): 6MP starting dose would only be reduced to 2/3rd, although not below 50 mg/m^2, when the pre-TEAM 6MP dose was > 50 mg/m^2/day - otherwise the patient continued on unchanged 6MP dosage. Furthermore, 6TG was initiated without the previous two weeks between 6MP dose reduction and initiation of 6TG therapy.
To ensure precise 6TG dosing to included patients, a liquid oral suspension of 6TG was manufactured at the Pharmacy at Rigshospitalet, University of Copenhagen, Copenhagen, Denmark, in accordance with good manufacturing process guidelines and with acceptable physical and chemical stability for up to 63 days.(5) Patients on TEAM received a 6TG suspension at least once monthly.

Dose limiting toxicities

In case of myelotoxicity (WBC < 1.5 x10^9/L, ANC < 0.5 x10^9/L or TBC < 50 x10^9/L) and/or hepatotoxicity (Alanine aminotransferase (ALAT) > 20 xUNL, bilirubin > UNL, coagulation factors II-VII-X < 0.50 IU/L or INR > 1.5), MTX/6MP/6TG therapy was paused. Handling of myelotoxicity was specified in December 2016; in case of WBC 1.0–1.5 x10^9/L, 6MP and MTX doses were reduced by 50%, and 6TG dosage was reduced according to a predefined algorithm (see below). When values had improved above/below these defined limits, MTX and 6MP were resumed at unaltered doses (i.e. 6MP/MTX doses administered before occurrence of the toxicity), whereas 6TG was resumed at a dose 2.5 mg/m^2/day below the toxicity-initiating level.
In case of three consecutive measurements of DNA-TG > 1500 fmol/µg DNA, 6TG dose was reduced by 2.5 mg/m^2/day. 6TG doses could subsequently be increased, if two consecutive measurements demonstrated DNA-TG < 1500 fmol/µg DNA. In case of SOS, all therapy would be discontinued and the patient would be excluded from further 6TG therapy.

Specification of handling of myelotoxicity, December 2016:
In case of WBC of 1.0-1.5 x10^9/L, 6MP and MTX doses were reduced by 50%, and 6TG dosage was reduced according to following algorithm.

6TG dose leading to WBC 1.0-1.5 x10^9/L	6TG dose should be reduced to
2.5 mg/m^2/day	0 mg/m^2/day (6TG completely withheld)
5 mg/m^2/day	2.5 mg/m^2/day
7,5 mg/m^2/day	2.5 mg/m^2/day
10 mg/m^2/day	5 mg/m^2/day
12.5 mg/m^2/day	7.5 mg/m^2/day

When values had improved above these defined limits, MTX and 6MP were resumed at unaltered doses, whereas 6TG was resumed at a dose 2.5 mg/m^2/day below the toxicity-initiating level.

5. Blood sampling and toxicity monitoring

All toxicities were registered in patients' medical file. Yet, a number of toxicities were so well-known and frequent during conventional MTX/6MP maintenance therapy that they were expected with current standard therapy regimens and were therefore not reported continually to the national regulatory authorities or to the Data Safety Monitoring Committee, including:
a. Since myelosuppression was the target therapy effect, leukopenia (the therapy monitoring parameter) would not be regarded as a SAE. This also included febrile neutropenia leading to hospitalization or prolongation of ongoing hospitalization if the patient's condition otherwise was good with no signs of septic shock.
b. Since myelosuppression was the target therapy effect, thrombocytopenia would not be regarded as a SAE.
c. A rise in ALAT with normal liver function tests (i.e. bilirubin and INR (or coagulation factor II-VII-X) is a well-known side effect of 6MP and would not be regarded as a SAE, unless in combination with abdominal problems indicating laparotomy.
d. A rise in bilirubin to less than 5 x UNL, unless in combination with other signs of SOS. SOS diagnosis according to criteria established by the Ponte Di Legno Toxicity Working Group.(2)
e. A decrease in coagulation factors II-VII-X, unless in combination with abdominal problems indicating laparotomy.
f. Infection/fever leading to hospitalization or prolongation of existing hospitalization.
g. Since the objective of the TEAM study was to increase DNA-TG levels, it would not be regarded as a SAE, if DNA-TG levels increased above 1500 fmol/µg DNA. However, DNA-TG levels > 1500 fmol/µg DNA (three consecutive measurements) resulted in 6TG dose reduction as described in the dose-limiting toxicities.

Clinical evaluation of patients during participation in the TEAM study
Included patients received a clinical examination with special focus on liver dysfunction at least biweekly, and more frequent if indicated. At each clinical examination blood samples were drawn to evaluate hematologic status (WBC including differential count), liver function (ALAT, coagulation factors II-VII-X, INR, bilirubin) and thiopurine metabolites, i.e. DNA-TG, erythrocyte TGN levels (Ery-TGN) and erythrocyte levels of methylated 6MP metabolites (Ery-MeMP). If MTX/6MP/6TG doses were adjusted to either obtain TEAM therapy targets or due to occurrence of a dose-limiting toxicity, blood samples were drawn after one week to reevaluate status, otherwise at least biweekly accompanied by clinical examination. Patients (and parents) kept a diary during the TEAM study to confirm drug doses and note survey symptoms.

6. Quantification of DNA-TG, Ery-TGN and Ery-MeMP

For DNA-TG quantification, 1−2 µg DNA was purified, depurinized, and ethenoderivatized with

chloroacetaldehyde. After normalization with their respective isotope internal standards, ratios of guanine and thioguanine were calculated. Ratios of guanine and thioguanine were measured with ultra-performance liquid chromatography tandem mass spectrometry and reported as fmol/µg DNA. With 1 µg DNA per sample, the limit of detection was at least 4.2 fmol/µg DNA and the limit of quantification at least 14.1 fmol/µg DNA, with intraday and interday relative SDs less than 11%, and analytical linearity up to a minimum of at least 10,000 fmol/µg.(6) A DNA-TG level of 100 fmol/µg DNA corresponds to a median incorporation ratio of approximately 1 TGN : 30,000 nucleobases.

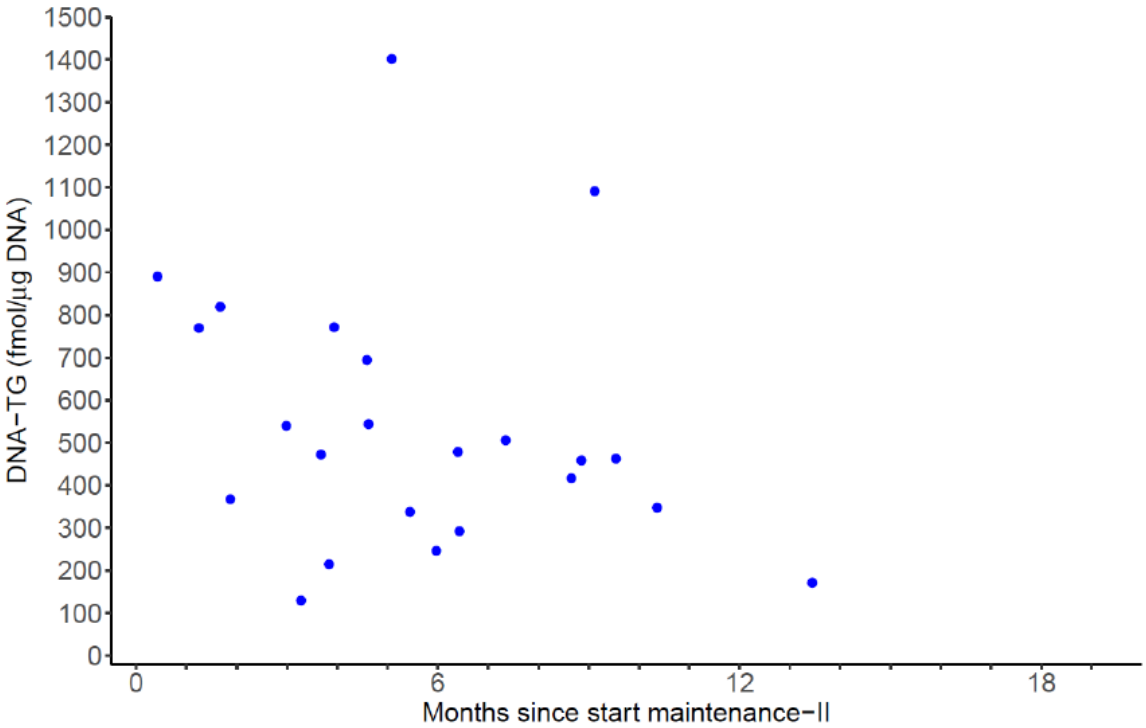
Quantification of thioguanine nucleotide concentration and level of methylated 6-Mercaptopurine metabolites in erythrocytes (Ery-TGN and Ery-MeMP respectively): We measured cytosolic metabolite concentrations of Ery-TGN and Ery-MeMP after protein precipitation and hot hydrolysis of whole blood with perchloric acid. We quantified the released thioguanine and 6MP nucleobases with ultraviolet ultra-performance liquid chromatography and normalized to hemoglobin (reported as nmol/mmol hemoglobin).(7)

Appendix 2: **Increments in DNA-thioguanine level during thiopurine enhanced maintenance therapy of acute lymphoblastic leukemia**
TEAM: Thiopurine Enhanced ALL Maintenance Therapy

Content:

1. **Power calculation for sample size for the TEAM study**
   Data presentation would be descriptive DNA-TG levels, tolerated dosage of 6-Thioguanine (6TG) and toxicities. The DNA-TG levels prior to 6TG therapy would be compared with the DNA-TG levels during TEAM therapy. From the NOPHO ALL-92 study we estimated the median DNA-TG level in ALL during the first four weeks of Methotrexate (MTX)/6-Mercaptopurine (6MP) maintenance-II to be 316.51 fmol/µg DNA based on measurements from 46 patients (unpublished data). The dynamic change in DNA-TG was analyzed log10-transformed and a linear mixed model used to determine the following parameters for the power calculation: 1) the increase in mean structure of log10-transformed DNA-TG = 0.00205 per week 2) intra class correlation (ICC) of 0.2 and 3) an inter-patient variation of 0.106. We expected an 80% increase from 300 fmol/µg DNA (current patient median) to 540 fmol/µg DNA after addition of 6TG to standard MTX/6MP maintenance-II. With 30 patients and 6 + 10 measurements before and after adding 6TG to 6MP/MTX, respectively, the power detecting such an increase in the median of log10-transformed DNA-TG is 99%.

2. **DNA-TG patient medians before TEAM. Supplemental Figure, Figure S1:**
   Time period before TEAM inclusion defined as two months prior to initiation of 6TG treatment. Blue dots mark individual DNA-TG patient medians before TEAM from each TEAM patient and timepoint of entry into the TEAM study.



3. **Number of patients included from the ALL2008 MT sub-study used for comparison with outcome parameters from the TEAM study.**
The historical data from the ALL2008 MT sub-study (patients who received standard Methotrexate(MTX)/6-Mercaptopurine(6MP) maintenance therapy) included measurements from 10 weeks after start of maintenance-II until discontinuation of antileukemic therapy.

Outcome	Number of patients	Mean number of samples pr. patient, IQR
DNA-TG	348	6 (2–10)
WBC	125	32 (24–43)
ANC	125	30 (19–41)
TBC	142	40 (29–52)
Ery-TGN	354	7 (2–11)
Ery-MeMP	334	7 (2–11)

IQR Interquartile range. **WBC** White Blood Cell Count, **ANC** Absolute Neutrophil Count, **TBC** Thrombocyte Count, **Ery-TGN** Thioguanine nucleotide level in erythrocytes, **Ery-MeMP** Methylated 6MP metabolite level in erythrocytes.

4. **Description of patients on TEAM therapy, who did not obtain DNA-TG target of 500 fmol/µg DNA**
Of the 32 patients who received at least 10 weeks of TEAM maintenance therapy, 27 patients obtained DNA-TG target above 500 fmol/µg DNA during TEAM therapy. Five patients did not obtain DNA-TG target. 1) One TPMT wildtype patient had prior to TEAM inclusion demonstrated poor MTX/6MP tolerance on an unresolved basis tolerating only 8 mg/m^2/week MTX and 23 mg/m^2/day 6MP. This led to insufficient myelosuppression (median WBC of 6.2 x10^9/L in the time period before TEAM inclusion) and low DNA-TG level (median 276 fmol/µg DNA before TEAM inclusion). Upon inclusion this patient had three months of remaining therapy. During TEAM therapy WBC and DNA-TG remained unchanged, and this patient reached maximum tolerated 6TG dose of 12.5 mg/m^2 just before discontinuation of

antileukemic therapy. If more time had been available, this patient potentially could have experienced an effect of TEAM therapy. 2) Another patient demonstrated an overall median DNA-TG level during TEAM therapy of 426 fmol/µg DNA, primarily reflecting a slow increase in DNA-TG at the start of TEAM therapy, as median DNA-TG was 870 fmol/µg DNA during the last five months of TEAM therapy. 3) One patient opted out of TEAM by parental decision after 33 weeks on TEAM therapy and likely did not experience the full effect of the TEAM strategy. 4+5) For two patients we found no apparent explanation for the low DNA-TG increment (24 and 77 fmol/µg DNA increment in DNA-TG during TEAM therapy, respectively).

5. **Thiopurine metabolite levels in erythrocytes.**
Figure S2a. Median thioguanine nucleotide level in erythrocytes (Ery-TGN) during TEAM therapy versus before TEAM. Each circle marks an individual patient's median Ery-TGN (nmol/mmol hgb) during TEAM therapy versus before TEAM. Black line represents the diagonal. **Figure S2b.** Median level of methylated 6-mercaptopurine metabolites in erythrocytes (Ery-MeMP) during TEAM therapy versus before TEAM. Each circle marks an individual patient's median Ery-MeMP level (nmol/mmol hgb) during TEAM therapy versus before TEAM. Black line represents the diagonal.

Figure S2a

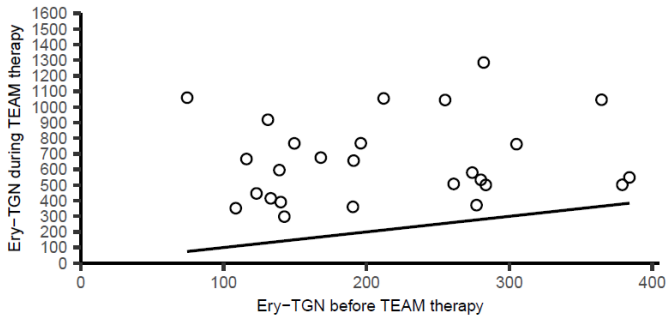


Figure S2b

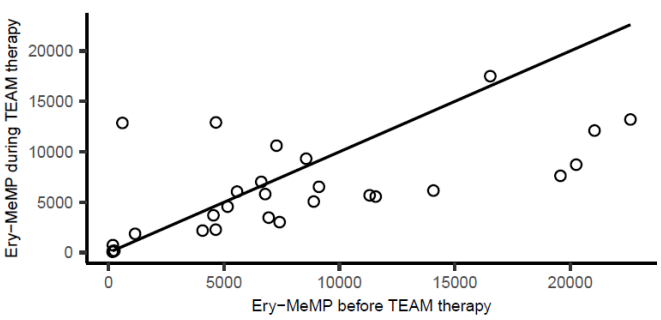
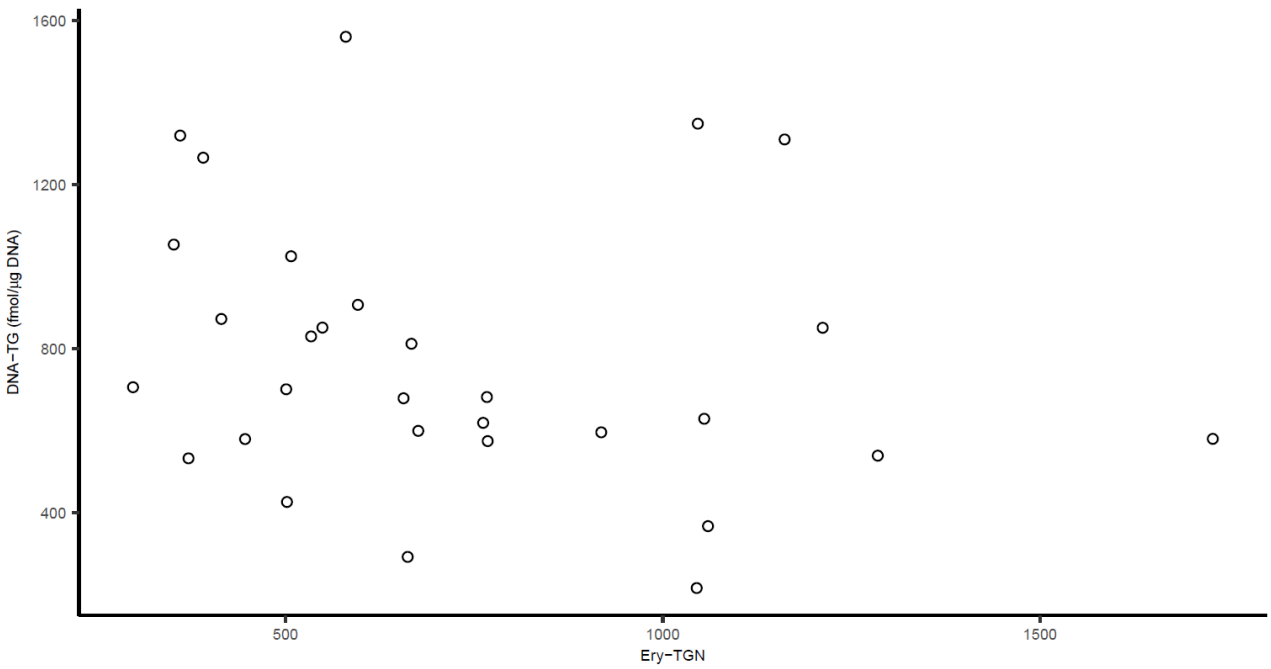


Figure S3. Plot of median Ery-TGN level during TEAM therapy in relation to median DNA-TG level during TEAM therapy.



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